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Note

Measurement of 3-methoxy-4-hydroxyphenylglycol sulfate ester in brain using reversed-phase liquid chromatography and electrochemical detection

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3-Methoxy-4-hydroxyphenylglycol (MHPG) is the major metabolite of norepinephrine (NE) in the mammalian central nervous system. This compound exists either in its free or conjugated forms (mainly as sulfate, MHPG-S) [1]. Its excretion in urine is supposed to reflect the activity of the central noradrenergic system in humans [2]. Changes in concentration of total MHPG or of MHPG-S are the best indicators of variations of NE turnover in the central nervous system. Several methods have been described to quantify the different forms of MHPG in tissue extracts and body fluids. MHPG-S can be hydrolysed to free MHPG either enzymatically or by acid hydrolysis. Free MHPG has been measured by gas chromatography with either flame-ionisation [3], electron-capture [4] or mass spectrometric [5] detection. These techniques require extraction with organic solvents, appropriate derivatization and need sophisticated equipment. A radioimmunological assay of MHPG of extremely high sensitivity has been described recently [6]; this assay has found limited utilization until now. The oldest method based on formation of a fluorophore of MHPG with ethylene diamine [7] lacked sensitivity in our hands. In recent years, methods based on liquid chromatographic separation combined with fluorimetric [8] and electrochemical [9] detection of MHPG have been developed.

We wish to describe here a method of measuring MHPG-S in brain extracts. This method is a new combination of known steps: brain extraction with H_2SO_4 [10], separation of MHPG-S from other constituents of brain extracts [7], hydrolysis to free MHPG by a purified preparation of *Helix pomatia* sulfatase [11], and quantification by high-performance liquid chromatography

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(HPLC) coupled with electrochemical detection [12]. This method is reliable, needs no extraction steps, and no sophisticated equipment in its manual version; 30—40 samples can be analysed by a single person in two working days.

MATERIAL AND METHODS

Reagents

The following chemicals were purchased: MHPG piperazine salt and yohimbine from Sigma, St Louis, MO, U.S.A.; MHPG-S as K^+ salt from Fluka, Buchs Switzerland; lyophilized "Helicase" (sulfatase + glucuronidase from *Helix pomatia*) from IBF, Clichy, France. Monofluoromethyl *p*-tyrosine methyl ester was synthesized in our laboratory by a method to be published elsewhere. All other reagents were of the highest analytical grade available from Merck, Darmstadt, F.R.G.

Liquid chromatography system

The liquid chromatography system contained the following components: a single piston pump (Kontron LC 410) fitted with a pulse dampener (Kontron Model 811) and set to deliver 1 ml/min, and a manual injector (Rheodyne 7125). The column was a LiChrosorb RP-18 (250 × 4.6 mm, 10 μm particle size) from Merck, fitted with a guard column (70 × 2 mm) filled with Co-Pell ODS (30 μm) from Whatman (Clifton, NJ, U.S.A.). The detector was a Model LC-4 electrochemical detector from Bioanalytical Systems (West Lafayette, IN, U.S.A.) with a glassy carbon electrode, the potential of which was set at 0.85 V versus the Ag/AgCl reference electrode. The signal was monitored on a dual-channel recorder (Omniscribe, Houston Instruments, Austin, TX, U.S.A.). The mobile phase was a mixture (89:11, v/v) of 0.15 *M* sodium dihydrogen phosphate and methanol containing 1.3×10^{-4} *M* EDTA; the pH was adjusted to 3.5 with H_3PO_4. Under these conditions, MHPG had a retention time of 6.8 min.

Purification of Helix pomatia sulfatase

It was necessary to purify the extract of *Helix pomatia* as there were a number of parasite peaks on the MHPG chromatogram with the crude extract. This was done by ion-exchange chromatography on DEAE-cellulose with a linear gradient of sodium chloride (0.03 *M* Tris—HCl pH 7.9; sodium chloride from 0 to 0.2 *M*) [11] (Fig. 1). Enzyme activity is determined with nitrocatechol sulfate as substrate. After dialysis of a pool of the most active fractions (hatched area in Fig. 1), the enzyme can be stored frozen at −20°C.

Hydrolysis of a standard solution of MHPG-S and stability of MHPG under hydrolysis conditions

To a solution of MHPG-S (250 ng of K^+ salt) in 3 ml of 0.25 *M* hydrochloric acid, an equal volume of 1 *M* sodium acetate is added so that the final pH is 5.5. Then 300 μl of purified sulfatase are added and the mixture is warmed to 37°C in a shaking water bath. A solution of MHPG piperazine is treated in the same way. Aliquots are withdrawn at given intervals. The enzyme is denatured

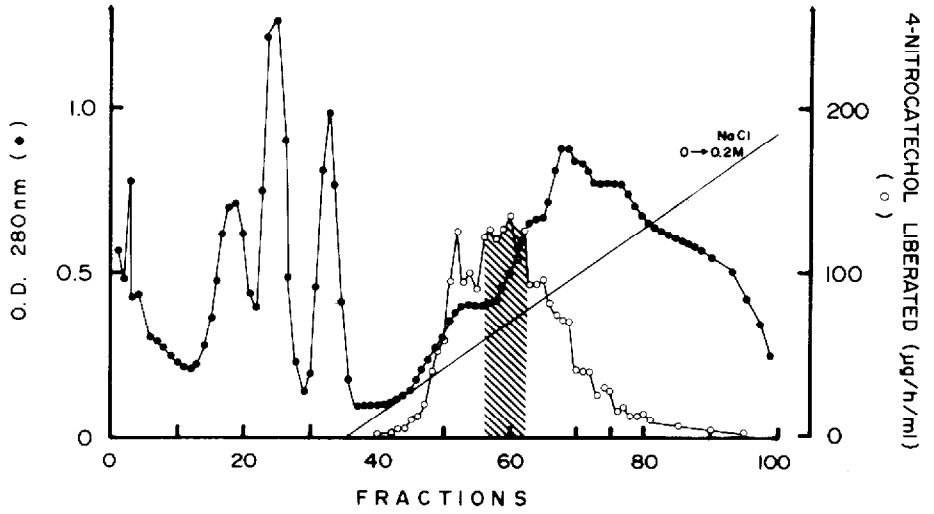

Fig. 1. Purification of the sulfatase from *Helix pomatia*. A lyophilized preparation of digestive juice of *Helix pomatia* (500 mg solubilized in 10 ml of 0.03 *M* Tris—HCl pH 7.9) was applied to a DEAE column equilibrated with the same buffer. The enzyme was eluted by a sodium chloride gradient as described in Materials and methods. The fractions corresponding to the hatched portion of the diagram were collected.

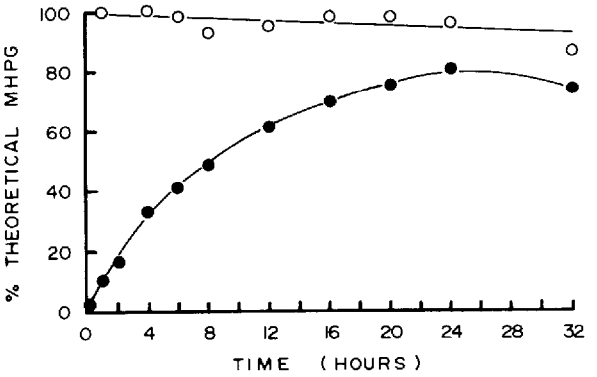

Fig. 2. Rate of hydrolysis of a standard solution of MHPG-S and stability of MHPG under hydrolysis conditions. MHPG-S and MHPG in 0.2 *M* sodium acetate pH 5.5 were allowed to react with a purified preparation of sulfatase (Fig. 1). Aliquots were analysed by HPLC combined with electrochemical detection at given time intervals.

by adding 0.1 volume of 1 *M* perchloric acid containing 1.5% $Na_2S_2O_5$. The amount of protein is so low that no centrifugation is needed before injection on the HPLC column. Fig. 2 shows the rate of MHPG appearance over 32 h. The hydrolysis levels off at 24 h and corresponds to 80% of the theoretical amount. Under the experimental conditions, the recovery of MHPG is superior to 90% after 36 h of incubation.

Tissue extraction and separation of MHPG-S

The procedure of Kohno et al. [10] was followed: homogenization in 0.1 *M* sulfuric acid, neutralization of the supernatant with 0.1 *M* barium

hydroxide up to pH 6.5. After centrifugation of the barium sulfate, MHPG-S was recoveered by ion-exchange chromatography on DEAE-Sephadex A_{25} as described by Meek and Neff [7].

Enzymatic hydrolysis of MHPG-S and HPLC of MHPG

To 3 ml of 0.25 *M* hydrochloric acid containing MHPG-S, 3 ml of 1 *M* sodium acetate are added to bring the pH to 5.5. This solution is incubated with 300 μl of purified sulfatase at 37° C for 24 h. After deproteinization of a 0.9 ml aliquot with 100 μl of 1 *M* perchloric acid containing 1.5% $Na_2S_2O_5$, the samples can be stored in the cold overnight; 25—50 μl are used for analysis in the HPLC system. Fig. 3 shows a typical chromatogram obtained with a standard solution of MHPG and a brain extract. There are no other peaks on the chromatogram so that the analysis time can be limited to 10—15 min. The amount of MHPG formed is calculated by comparing its peak heights to that of the known standard.

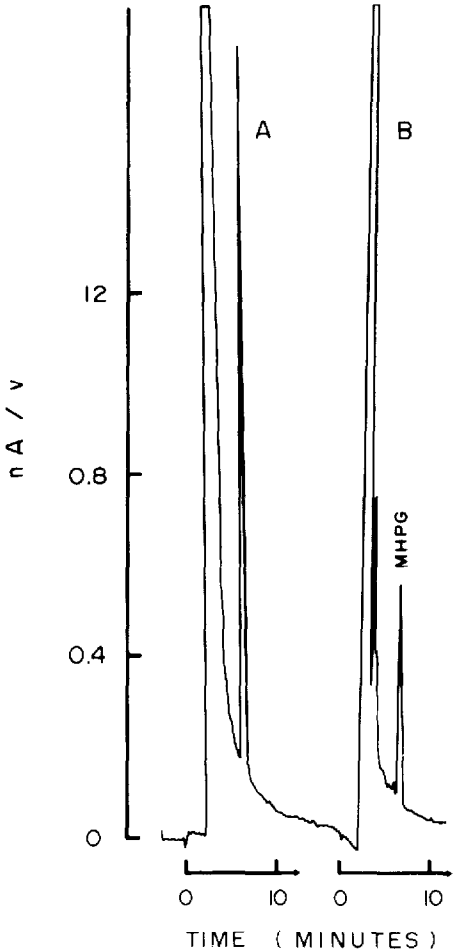

Fig. 3. Chromatogram of a standard solution of MHPG and of a brain extract after hydrolysis. (A) Chromatogram of a standard solution of MHPG. The peak corresponds to an injection of 1 ng. (B) Chromatogram of a brain extract processed as described in Materials and methods. The injection volume was 20 μl; the peak corresponds to 0.28 ng of MHPG.

Calculation of total recovery and reproducibility

A pool of five rat brains was homogenized in 40 ml of 0.1 *M* sulfuric acid. This pool was distributed into 4-ml aliquots, to which different amounts of commercial MHPG-S were added. The samples were processed as described above. There is a linear relationship between the added MHPG-S and the recovered MHPG. The regression line was calculated with a least-squares program on a Hewlett-Packard 9820A calculator: $y = 0.368x + 193$ ($r = 0.995$, $p \leqslant 0.01$).

To test day-to-day reproducibility the recovery was calculated after addition of 992 pmol of MHPG-S to brain extracts on consecutive days. The mean recovery from seven experiments was 369 ± 19 pmol, i.e. 37 ± 2%. No attempt was made to identify the steps responsible for the major loss.

RESULTS

The method of measuring MHPG-S has been applied to quantify brain concentrations of this NE metabolite in control animals, under conditions of increased NE turnover, and under conditions of inhibition of NE synthesis (Table I). Control values calculated by this method are 188 ± 6 ng/g (mean ± S.E.M. $n = 5$). Values in the literature for whole rat brain range from 40 to 60 ng/g ([1], unspecified strain) to 149 ng/g ([10], male Wistar). Our values for Sprague-Dawley are some 20% superior to this last figure. Yohimbine produces an increase of NE outflow and synthesis presumably by blocking presynaptic α_2-receptors. As expected and as reported previously [13], MHPG-S levels are almost doubled in animals treated with 2.5 mg/kg yohimbine. We reported recently that monofluoromethyl *p*-tyrosine methyl ester produces a selective inhibition of catecholamine synthesis in rat brain [14]. We believe that the compound is hydroxylated by the action of tyrosine hydroxylase to monofluoromethyl-DOPA, a potent irreversible inhibitor of aromatic amino acid

TABLE I

BRAIN CONCENTRATION OF MHPG-S IN CONTROL ANIMALS AND UNDER CONDITIONS OF STIMULATION AND INHIBITION OF NE SYNTHESIS

Groups of rats (male Sprague—Dawley, 150 g) were treated as follows. One group received saline intraperitoneally and served as control. A second group was injected intraperitoneally with 2.5 mg/kg yohimbine. A third group was given by gavage 100 mg/kg monofluoromethyl *p*-tyrosine methyl ester (in 1% ascorbic acid). The last group received both yohimbine and the tyrosine analogue simultaneously. The animals were killed 4 h after the different treatments; the brains were split sagitally. One half was processed as described for MHPG-S determinations, the other half was saved for other purposes. Each value represents the mean ± S.E.M. of 5 animals.

	Brain MHPG-S (ng/g)
Control	188 ± 6
Yohimbine	360 ± 20*
Monofluoromethyl *p*-tyrosine	148 ± 10*
Yohimbine + monofluoromethyl *p*-tyrosine	205 ± 3

*$p < 0.01$ (Student's *t*-test compared to control).

decarboxylase (AADC) [15]. Therefore the inhibitory effect, both on AADC and on amine synthesis, should depend on activation of the tyrosine hydroxylase [14]. As reported in Table I, the compound has by itself only a small, albeit significant, effect on brain MHPG-S concentration. However, when given together with yohimbine, monofluoromethyltyrosine blocks almost completely the accumulation of MHPG-S due to α_2 blockade.

DISCUSSION

The method of MHPG-S determination described here combines a series of steps described previously. The extraction with sulfuric acid was found to be more reproducible than that with zinc sulfate. Precipitation by barium hydroxide was best performed on a supernatant rather than on the homogenate as the pH can be adjusted more easily. The isolation of MHPG-S by DEAE-Sephadex had been used by Meek and Neff [7] in their original method. The extract of a 1-g brain sample can be applied in totality to 0.75 ml of resin, so that no correction of volume is needed up to that stage. There is no loss in the washing of the resin with the 0.06 *M* hydrochloric acid and release is quantitative in the subsequent step. In our hands, acid hydrolysis of the eluted MHPG-S by hydrochloric acid or perchloric acid gave low yields and produced an erratic peak interfering with MHPG. For reasons already explained we found it absolutely necessary to purify the enzyme for enzymatic hydrolysis. As can be seen in Fig. 3, the chromatogram shows no other peaks than MHPG. The chromatographic conditions were modified from those used by Wagner et al. [12] to measure catecholamines and their metabolites in cerebrospinal fluid. Addition of $Na_2S_2O_5$ results in a stable baseline which increases the sensitivity of the detection. With adaptation of extraction, elution and hydrolysis volumes, the method should be applicable to measure regional distribution of MHPG-S. Although relatively low, the recovery of MHPG-S is reproducible from sample to sample and from day to day. This method has allowed us to confirm that the α_2-antagonist yohimbine produced an accumulation of MHPG-S in rat brain and that the inhibitory effect of monofluoromethyl *p*-tyrosine on NE synthesis is enhanced under conditions which increase NE turnover.

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